MORPHOLOGICAL EFFECTS OF COBALTOUS CHLORIDE ON THE DEVELOPMENT OF LIMNAEA STAGNALIS AND LIMNAEA PALUSTRIS[1]

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Numerous investigators have reported the effects of altering the environment of developing embryos, especially echinoderms, with divalent ions of cobalt, nickel and zinc, the transition metals. Echinoderm eggs exhibit hypodevelopment of the endoderm and mesoderm after treatment with these ions. Exogastrulae and abnormal pluteus larvae with reduced arms, skeletal rods, and gut commonly result (Waterman, 1937; Rulon, 1953, 1955, 1956, 1957; Lallier, 1955a, 1955b, 1956, 1959; Mateyko, 1961). A pronounced animal-vegetal gradient and a developmental requirement for a salt-containing medium characterize the echinoderm egg. A mosaic egg, capable of development in a de-ionized environment, might be expected to respond differently to transition metals. In the absence of published information concerning the effect of transition metals on such eggs, a study was made of the morphogenetic effects of one transition metal, cobalt, on the egg of *Limnaea,* a fresh-water pulmonate snail.

Materials and Methods

The initial experiments were performed in Utrecht, the Netherlands, with eggs of *L. stagnalis;* in the latter experiments, eggs of the American species, *L. palustris,* were used.

Encapsuled eggs were treated according to the standard conditions of Raven (1956) and Geilenkirchen (1961), to permit comparison with previous studies on *Limnaea.* When the eggs reached the two-cell stage (stages 3 to 6 of Raven, 1946), the egg capsules were freed from the surrounding jelly and divided into lots of 20. The 24-hour treatment was begun at this stage or 24 or 48 hours later. During the treatment period the eggs were incubated at 25° C. in shell vials with various concentrations of $CoCl_2$ in 10 ml. of distilled water. Control eggs were treated similarly but incubated in pond water or distilled water of pH 6–7. Eggs treated during the later periods developed first in tap or pond water or on 2% agar plates.

Embryonic development was studied from whole mounts and serial sections made at 24-hour intervals from the end of the treatment until the normal embryos were larval snails (6–8 days after oviposition). Whole mounts were prepared of embryos fixed in Bouin's solution and cleared in wintergreen oil. Serial sec-

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tions were prepared of embryos fixed in Bouin's solution, sectioned at 6–8 micra and stained with Ehrlich's or Heidenhain's hematoxylin and eosin.

Results

The morphological effects of 24-hour treatment of *Limnaea* eggs or embryos with $CoCl_2$ (1×10^{-4} *M* to 2×10^{-6} *M*) are given in Table I (*L. stagnalis*) and Table II (*L. palustris*). The histological and morphological characteristics of these malformations are described in subsequent sections.

Table I summarizes the results of ten experiments on eggs of *L. stagnalis* treated during the first 24 hours of development and three experiments on em-

Table I

Morphological effects of cobaltous chloride on the development of eggs and embryos of Limnaea stagnalis

Treatment		No. of eggs	Effect of Treatment					
							Snail	
Treatment period	Conc. $CoCl_2$ (*M*)		Direct mortality %	Exogastrula %	Gastrula %	Veliger %	Shell malformations %	Normal %
First 24 hours	1×10^{-4}	166	82.5	11.4	4.8	1.3	—	—
	8×10^{-5}	160	64.4	9.4	10.6	14.4	—	1.2
	4×10^{-5}	325	29.5	7.7	11.7	39.8	10.5	0.8
	3×10^{-5}	179	21.2	16.2	12.9	30.8	4.9	14.0
	1×10^{-5}	196	10.2	6.6	1.5	6.6	8.7	66.4
	0	288	2.0	—	—	—	—	98.0
Second 24 hours	1×10^{-4}	60	65.0	—	23.0	8.0	—	—
	5×10^{-5}	60	35.0	—	61.0	4.0	—	—
	1×10^{-5}	60	21.6	—	—	78.4	—	—
	0	60	3.3	—	—	—	—	96.7
Third 24 hours	1×10^{-4}	57	90.0	—	10.0	—	—	—
	5×10^{-5}	57	86.0	—	10.0	4.0	—	—
	1×10^{-5}	57	51.0	—	1.0	49.0	—	—
	0	60	—	—	—	—	—	100.0

bryos treated during the second (gastrula to trochophore stage of Raven, 1946) and third (late trochophore to early veliger stage of Raven, 1946) 24-hour period. Concentrations of $CoCl_2$ greater than 10^{-4} *M* killed the embryos directly; at 10^{-4} *M* few survived the treatment period and none developed to the snail stage ("hippo" stage of Raven, 1949). Treatment with lower concentrations of $CoCl_2$ (8×10^{-5} to 1×10^{-5} *M*) during the first 24-hour period reduced direct mortality and increased the numbers of normal snails. The incidence of exogastrulae and arrested gastrulae was approximately constant at all but the lowest concentration tested; arrested veligers and shell-less snails were most frequent with 4×10^{-5} *M* $CoCl_2$. None of the embryos treated during the second and third 24 hours developed beyond the veliger stage. Those which were not killed during the treatment

period were arrested at the pre-veliger stage or developed into typical arrested veligers with reduced larval liver, foot and shell. The reduction of $CoCl_2$ concentration from 5×10^{-5} *M* to 1×10^{-5} *M* increased ten-fold the incidence of arrested veligers among the group treated during the second 24 hours. It appears that this method might be used to produce arrested veligers selectively, as the yield approached 80%. The results in Table I suggest that *L. stagnalis* embryos may be more sensitive to cobalt during the third than during the first or second 24 hours of development.

TABLE II

Morphological effects of cobaltous chloride on the development of eggs and embryos of Limnaea palustris

Treatment		No. of eggs	Effect of Treatment						
							Snail		
Treatment period	Conc. $CoCl_2$ (*M*)		Direct mortality %	Exogastrula %	Gastrula %	Veliger %	Shell-less %	Helmet %	Normal %
First 24 hours	8×10^{-5}	200	81.0	12.0	5.0	2.0	—	—	—
	4×10^{-5}	200	33.5	55.0	5.0	5.0	1.0	—	—
	2×10^{-5}	120	4.2	71.5	3.3	15.8	3.3	0.8	—
	1×10^{-5}	200	12.5	20.5	7.0	29.5	17.5	0.5	9.5
	6×10^{-6}	170	4.7	4.7	—	18.8	24.2	21.2	26.4
	2×10^{-6}	120	2.5	—	—	5.0	20.0	7.5	65.0
	0	200	—	—	—	—	—	1.5	98.5
Second 24 hours	8×10^{-5}	120	93.3	—	—	6.7	—	—	—
	4×10^{-5}	120	81.7	—	—	15.0	3.3	—	—
	1×10^{-5}	120	65.0	—	—	21.3	13.3	—	—
	6×10^{-6}	120	6.7	—	—	6.7	45.0	6.7	33.9
	2×10^{-6}	120	2.1	—	—	3.0	13.3	5.0	76.6
	0	120	—	—	—	—	—	—	100.0
Third 24 hours	8×10^{-5}	120	90.0	—	—	6.6	—	3.4	—
	4×10^{-5}	120	75.0	—	—	—	10.0	—	15.0
	1×10^{-5}	120	13.4	—	—	5.0	10.0	—	71.6
	6×10^{-6}	120	6.6	—	—	—	—	13.4	80.0
	2×10^{-6}	120	16.7	—	—	—	—	—	83.3
	0	120	10.0	—	—		3.4	—	86.6

Table II summarizes the results of ten experiments on *L. palustris* embryos treated during the first 24 hours and six experiments on embryos treated during the second (trochophore to early veliger stage) and third (early to mid-veliger stage) 24 hours of development. The effective concentrations of $CoCl_2$ ranged from 2×10^{-6} to 8×10^{-5} *M*. Concentrations greater than 4×10^{-5} *M* killed most of the eggs. The maximal percentage of exogastrulae resulted when embryos were treated during the first 24 hours with 2×10^{-5} *M* cobalt. The maximum number of arrested veligers and shell-less snails was obtained following treatments during the first or second 24 hours with 10^{-5} and 6×10^{-6} *M* cobalt. Exogastrulae and arrested gastrulae were absent in embryos treated after the first 24 hours, since the embryos had gastrulated by then. In contrast to the results

with *L. stagnalis, L. palustris* embryos were most resistant to exposure to $CoCl_2$ during the third day of development, since normal snails were obtained following treatment with concentrations as high as 4×10^{-5} *M*. During the first 24 hours *L. stagnalis* appeared to be more resistant than *L. palustris,* but the situation was reversed during the second and third periods. These differences are probably due to differences in rate of development; *L. palustris* develops faster than *L. stagnalis* and may have passed the sensitive stages by the third 24 hours. There does not appear to be any critical period of cobalt sensitivity.

Both species of eggs varied considerably in resistance from one experiment to the next and within one experiment. Seasonal changes in the physiological sensitivity (Haije and Raven, 1953; Raven, 1956; Geilenkirchen and Nijenhuis, 1959) cannot fully explain the variations in sensitivity in the present study, as they were observed also among eggs from the same egg mass.

Morphology of malformations

Seven grades of developmental disturbances, ranging from death to slowed normal development, were observed in treated embryos of *L. stagnalis* and *L. palustris*.

Direct mortality. This category includes embryos which died during the treatment period. Eggs killed during the first 24 hours were usually arrested in the early cleavage and pre-gastrula stages. The blastomeres tended to be spherical and to separate from one another, especially at the eight-cell stage, where the four macromeres often had separated from one another but remained attached to the first quartette of micromeres. This condition suggests a preferential loss of cell adhesiveness at the vegetal pole of the embryo. In several experiments the separated macromeres of *L. palustris* eggs treated with 4×10^{-5} *M* $CoCl_2$ were the same size as the micromeres, indicating that the third cleavage plane had been depressed toward the vegetal pole. The cells of embryos killed during the second and third 24-hour treatment periods also tended to be spherical and to separate from one another.

Exogastrulae. Embryos arrested at the gastrula stage frequently developed into vesicular exogastrulae with subpressed invagination of the archenteron (Raven, 1942). They often swelled into hydropic vesicles with a group of endodermal cells visible in the blastopore region (Fig. 1). Such vesicles usually died within a few days, but an occasional one lived as long as 10 days. Dumbbell-shaped exogastrulae (Fig. 2) occurred less frequently. Occasionally a dumbbell-shaped exogastrula would separate into two vesicles; neither developed further.

Arrested gastrulae. This group includes embryos that gastrulated but were one-half to two-thirds the size of the control gastrulae. A few developed as far as the trochophore stage of Raven (1946); none developed into veligers.

Arrested veligers. The most conspicuous morphological malformation occurred at the veliger stage (Raven, 1946), when the normal 4-day embryo or veliger (Fig. 6) had the following features: a distinct head, foot, and body region; paired cephalic plates; a stomodeum and radula sac; a shell and mantle fold covering most of the body; and a body lined with a cup-shaped mass of large endoderm cells. The large endoderm cells formed the larval liver or digestive gland and surrounded the small endoderm cells of the developing mid-gut. The cobalt-

arrested veligers (Figs. 3, 4, 5) were smaller than the normal 4-day veliger. Their shells were limited to the area of the shell gland primordium on the left posterior side of the embryo; the larval liver cells formed a compact mass of cells separated from the body wall by a fluid-filled space that extended into a rudimentary foot. The stomodeum, radula sac (Fig. 3) and paired cephalic plates

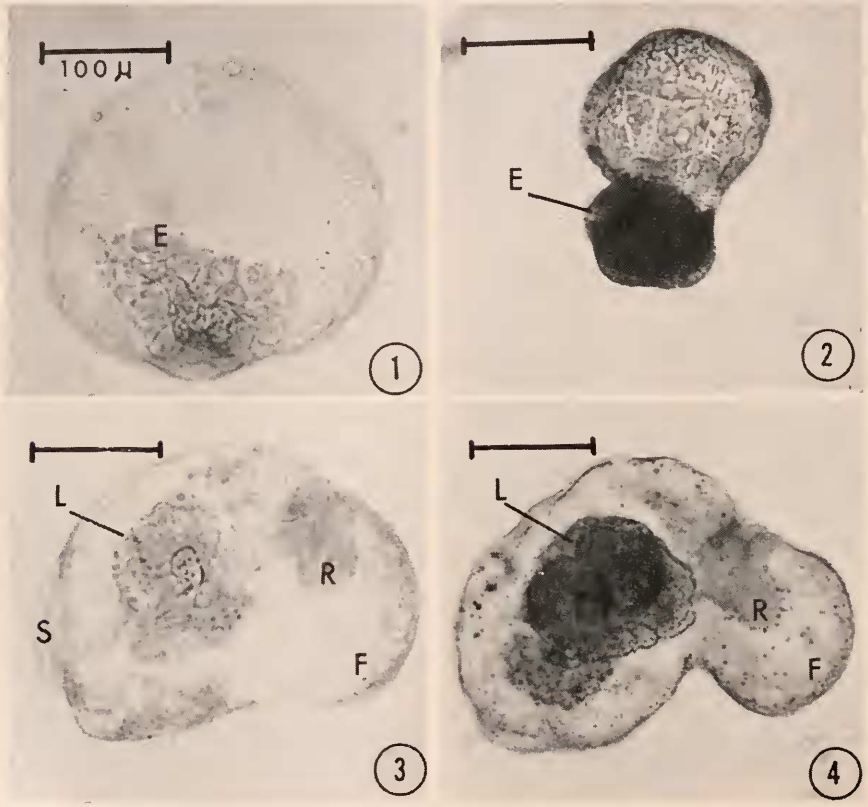

FIGURE 1. Whole mount of an 8-day-old hydropic exogastrula with invaginated mass of endoderm cells.

FIGURE 2. Whole mount of a 4-day-old dumbbell-shaped exogastrula with invaginated endoderm.

FIGURES 3 and 4. Whole mounts of 8-day-old cobalt-arrested veligers with reduced larval liver mass.

Key for Figures 1 to 4. E, endoderm; F, foot; L, larval liver; R, radula sac; S, shell gland.

usually were visible. Some arrested veligers became hydropic, showing a swollen foot or body. The vesicular condition of the body apparently resulted from a reduction in the number and size of the large endoderm cells. The vesicular foot was associated with a reduction in mesenchyme tissue. Arrested veligers usually lived for 3–6 days without undergoing further differentiation. The cobalt-arrested veligers were similar morphologically to "aspecific snails" obtained from

centrifuged eggs (Raven and Tates, 1961) and to embryos classified under "other malformations" obtained by lithium treatment (Geilenkirchen and Nijenhuis, 1959).

Shell-less snails. These embryos developed as far as the hippo stage (Raven, 1942), had a pulsating heart and paired eyes and tentacles. They were usually smaller than the control embryos and had the following disturbances: a shell limited to the shell gland area, a relatively small mass of larval liver cells in the center of the body, an occasional hydropic foot or body. These snails never hatched from their capsules.

Helmet-shelled snails. A few embryos developed into snails that were normal except that the shell had an abnormally wide aperture. These resembled the

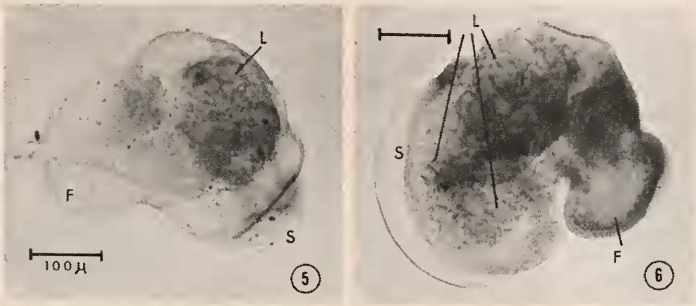

FIGURE 5. Whole mount of an 8-day-old cobalt-arrested veliger.
FIGURE 6. Whole mount of a normal 4-day-old veliger.
Key for Figures 5 and 6. F, foot; L, larval liver; S, shell gland and shell.

helmet-shelled snails Raven and Spronk (1952) produced with beryllium chloride treatment.

Normal snails. Snails which developed from this group were morphologically similar to the controls, although in most cases they developed more slowly.

Histological malformations

Arrested and normal veligers. Histological sections were prepared of normal 3- and 4-day embryos and of 8-day arrested veligers of *L. stagnalis*, to determine the extent of endo-mesodermal differentiation in the cobalt-arrested veligers. The normal 3-day *L. stagnalis* embryo possessed the shell gland, stomodeum and radula sac (Fig. 7). The large endoderm cells of the larval liver were numerous, densely eosinophilic, and closely juxtaposed to the ectoderm, occupying most of the body cavity. At this stage the foot was only a bulge ventral to the stomodeum. Sections of the normal 4-day *L. stagnalis* embryo (Fig. 8) revealed that the larval liver cells lined most of the body proper and were arranged in one to two cup-shaped layers surrounding the mid-gut. The small-celled endoderm was differentiated into mid- and hind-gut. The shell gland had developed into the mantle fold surrounding the posterior half of the body. The foot was distinct and filled with mesenchyme cells. The tentacle *anlagen* were present. Comparison of Figures 7 and 8 shows that the 4-day embryo is almost twice as large as the

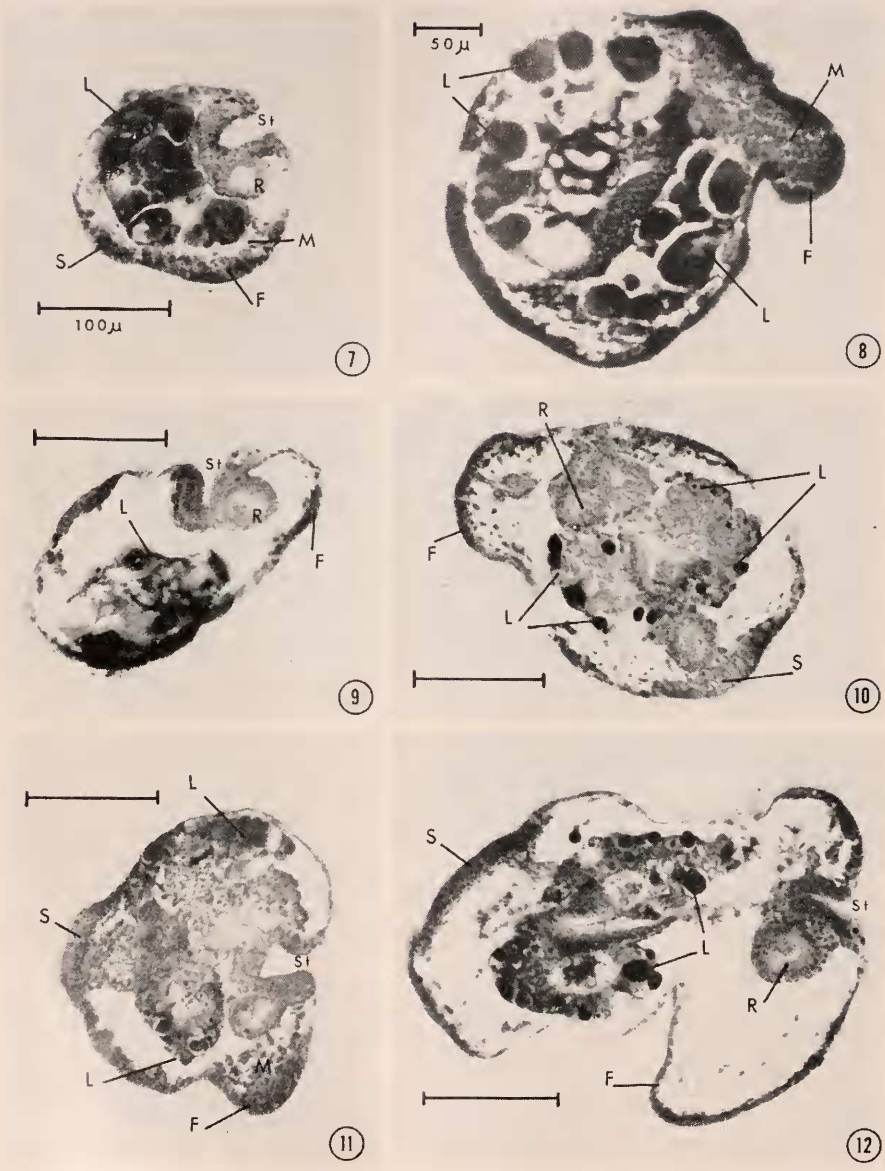

FIGURE 7. Sagittal section of a normal 3-day-old embryo or late "trochophore."

FIGURE 8. Sagittal section of a normal 4-day-old embryo or "veliger."

FIGURE 9. Sagittal section of an 8-day-old arrested veliger treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 10. Sagittal section of an 8-day-old arrested veliger treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 11. Sagittal section of an 7-day-old arrested veliger treated the first 24 hours with 3×10^{-5} *M* $CoCl_2$.

FIGURE 12. Sagittal section of an 8-day-old arrested hydropic veliger treated the first 24

3-day embryo, indicating that during the third day of development there is considerable growth accompanying the initial phases of organogenesis.

Sections of 8-day arrested veligers of *L. stagnalis* (Figs. 9, 10, 11) revealed that the stomodeum and radula sac were similar to those of the normal 3-day embryo, and that the small-celled endoderm had formed a mid-gut that in some embryos was continuous with the stomodeum. Therefore, these embryos were morphologically equipped to ingest nutrients from the surrounding capsule fluid. However, the larval liver cells, the main site of capsule fluid digestion at this stage (Bloch, 1938; Raven, 1958), were abnormally small, few in number, and irregularly eosinophilic (Figs. 9–12). The relatively large space between the larval liver and body wall and the foot contained few mesenchyme cells; the hydropic veligers were especially deficient in mesenchyme cells (Fig. 12). The mantle fold was limited to the region of the shell gland at the posterior end of the larva.

In summary, observations on whole mounts and sections showed that arrest at the veliger stage entailed inhibition of the differentiation and proliferation of mesenchyme cells of the body and foot, and inhibition of proliferation and enlargement of the endoderm cells of the larval liver.

Treated and normal 1-, 2- and 3-day embryos. In order to determine the degree of endodermal and mesodermal differentiation prior to the appearance of exogastrulae and arrested veligers, sections were prepared of 1-, 2- and 3-day-old *L. stagnalis* embryos. The treated group had been exposed to 4×10^{-5} *M* $CoCl_2$ for the first 24 hours.

The lumen of the blastocoel of the normal 1-day embryo was characteristically filled with mesomeres containing darkly staining cytoplasm and many γ granules (Fig. 13). There were fewer mesomeres and a larger blastocoel in the cobalt-treated embryos (Fig. 14). Neither normal nor treated embryos had begun to gastrulate.

Treated and normal 2-day-old embryos had gastrulated (Figs. 15, 16). In the normal gastrulae the blastocoel was obliterated by the invaginated endomeres (Fig. 15). Furthermore, there was intimate contact between the endomeres at the tip of the archenteron and the overlying ectomeres, and also between endomeres, mesomeres and ectomeres, as had been previously reported (Raven, 1952a). The endoderm cells lining the archenteron contained material that stained as the capsule-fluid albumen trapped in the archenteron. In the cobalt-treated embryos the mesomeres were limited to a few cells at the vegetal end of the blastocoel, which was not filled with cells. The endomeres had invaginated and their protoplasmic processes extended toward the inner side of the ectoderm (Fig. 16); the failure of the endomeres to make contact with the ectomeres may be due to a fixation artifact. If the cobalt-treated embryo sectioned for Figure 16 had been allowed to develop, it probably would have exogastrulated or developed into an abnormal veliger.

Normal 3-day embryos had reached the late "trochophore" stage (Raven, 1946) (Figs. 7, 17); the shell gland was present and was in intimate contact with the

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hours with 4×10^{-5} *M* $CoCl_2$. Body and foot collapsed during the fixation and dehydration process.

Key for Figures 7 to 12. F, foot; L, larval liver; M, foot mesenchyme; R, radula sac; S, shell gland; St, stomodeum.

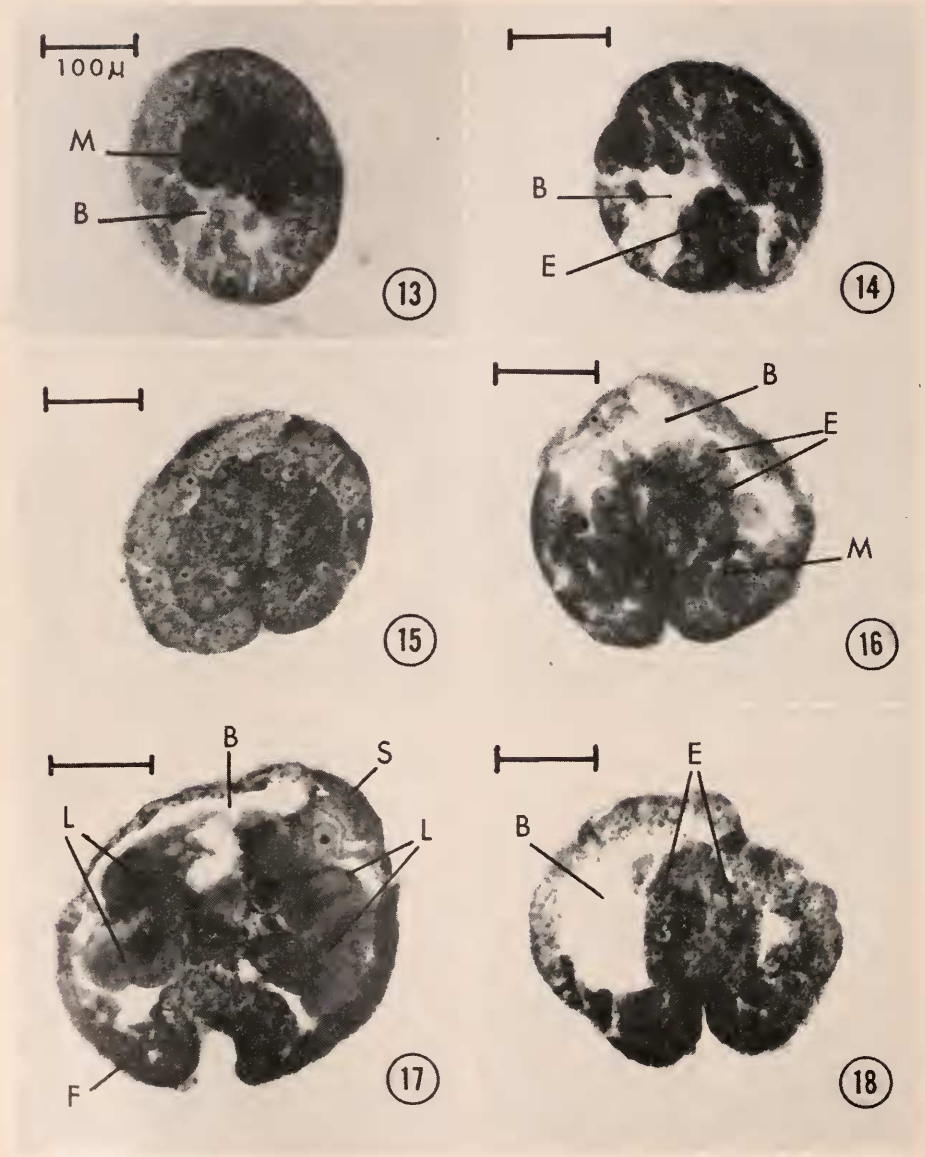

FIGURE 13. Section of a normal 1-day old embryo.

FIGURE 14. Sagittal section of an abnormal 1-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 15. Sagittal section of a normal 2-day-old embryo.

FIGURE 16. Sagittal section of an abnormal 2-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

FIGURE 17. Sagittal section of a normal 3-day-old embryo.

FIGURE 18. Sagittal section of an abnormal 3-day-old embryo treated the first 24 hours with 4×10^{-5} *M* $CoCl_2$.

Key for Figures 13 to 18. B, blastocoel or body cavity; E, endoderm cells; F, foot primordium; M, mesoblasts; L, larval liver cells; S, shell gland primordium.

small-celled endoderm. The stomodeum was differentiated into an oral cavity and a radula sac. Large albumen-filled larval liver cells filled most of the body cavity and extended anteriorly between the radula sac and foot *anlagen*, which consisted of a small bulge of ectoderm that had proliferated several layers of cells. Spindle-shaped mesenchyme cells were scattered in the body cavity between the larval liver cells and the body wall ectoderm. The treated 3-day embryos were smaller than the controls, lacked a stomodeum and radula sac, had few mesenchyme and few larval liver cells (Fig. 18). However, as Figure 18 shows, there was intimate contact between the tip of the archenteron and the overlying ectoderm.

Sections of 1-, 2- and 3-day-old cobalt-treated embryos support the idea that cobalt inhibits the proliferation of the mesomeres and growth of the larval liver cells. The reduction in size and number of these two types of cells results in (1) a partially cell-filled blastocoel cavity following gastrulation, and (2) a lack of intimate contact between the endodermal and mesodermal cells and the ectodermal cells. Continued ectodermal proliferation and uptake of water or other fluids by the blastocoel may cause formation of a vesicular or dumbbell-shaped exogastrula. In less extreme instances the abnormal embryo might differentiate into an abnormal veliger or snail.

24-hour treatment with other metal salts

Lallier (1955a, 1955b) and Rulon (1953, 1955, 1956, 1957) showed that chloride salts of cadmium, zinc, nickel, manganese and cobalt caused similar

Table III

Effective molar concentrations of cobaltous chloride and other metal chlorides on L. palustris eggs

Salt	Maximum direct mortality	Maximum arrested veligers	Maximum normal snails
$MnCl_2$	5×10^{-5} *M*	2×10^{-5} *M*	1×10^{-5} *M*
$CoCl_2$	4×10^{-5} *M*	1×10^{-5} *M*	2×10^{-6} *M*
$CdCl_2$	6×10^{-6} *M*	2×10^{-6} *M*	1×10^{-6} *M*
$NiCl_2$	8×10^{-6} *M*	4×10^{-6} *M*	2×10^{-7} *M*

morphological abnormalities in echinoderm embryos. In the present study a series of experiments was run to test the morphogenetic activity of cadmium, nickel and manganese ions on *L. palustris* eggs. All three ions produced morphological abnormalities similar to the abnormalities obtained following cobaltous chloride treatment. The minimum concentrations of these several ions and of cobalt which produced the maximal direct mortality, maximal arrested veligers, and maximal normal snails are summarized in Table III. The sequence of effective concentrations is Mn < Co < Cd < Ni.

Discussion

Cobaltous chloride causes several characteristic malformations in *Limnaea;* the cobalt-sensitive elements appear to be the fourth quartette of micromeres and their derivatives, the mesomeres and larval liver cells. A variety of agents including

Ni^{++}, Cd^{++}, Mn^{++}, alkali metals, alkaline earth metals, centrifugation, and heat shock produce similar abnormalities (Raven, 1942, 1952a, 1956; Raven and Dudok de Wit, 1949; Raven and Kovoets, 1952; Raven and van Egmond, 1951; Raven and van Erkel, 1955; Raven and Spronk, 1952; Raven *et al.*, 1947; Geilenkirchen, 1961; Geilenkirchen and Nijenhuis, 1959). Exogastrulae, arrested veligers, and hydropia also occur in eggs laid and developed in aquaria, and are common in eggs collected in natural habitats.

Exogastrulae and hydropia

Exogastrulation in *Limnaea* has been ascribed to nonspecific action on the material of the vegetal hemisphere (Raven, 1952a, 1952b), to injury of the cortical factors involved in ooplasmic segregation (Raven, 1958) and to interference with the differentiation and invagination of the endomeres, causing accumulation of fluid in the blastocoel (Geilenkirchen and Nijenhuis, 1959). The latter authors also suggest that impairment of osmoregulatory mechanisms, such as the larval kidneys, may partially be responsible. The observations presented in this paper suggest that inhibition of proliferation and differentiation of the mesomeres may be the cause of exogastrulae and hydropia.

Raven (1946, 1958) reported that gastrulation in *Limnaea* occurs by invagination of the endomeres. By means of pseudopodia the endomeres connect with the inner side of the cells of the animal hemisphere and with the mesomeres occupying the ventral, posterior and lateral regions of the blastocoel (Fig. 15; Raven, 1946). Cobalt treatment appears to suppress the formation of the primary mesoblasts (Figs. 14, 18). As a result there are relatively few mesomeres present in the blastocoel at the time of endomere invagination; the surfaces of the mesomeres may be altered, reducing also the affinity and adhesion between the mesomeres and ectomeres. Nevertheless, the endomeres sometimes do invaginate (Figs. 16, 18), but their pseudopodia do not always effect contact with the ectomeres across the abnormally large blastocoel (Fig. 16). Despite the lack of mesomeres, invagination may be completed if contact between the endomeres and ectomeres is established (Fig. 18). During this process, the blastocoel swells, probably because colloidal material accumulated in the blastocoelic fluid causes increased water uptake. When the gastrula hatches from the vitelline membrane, the fluid-filled space may enlarge further, producing a hydropic vesicular exogastrula.

Inhibition of proliferation and differentiation of the mesomeres may also be responsible for the hydropic foot and body cavity of larvae and shell-less snails. Some arrested larvae appeared to have more mesenchyme than others, particularly in the foot region; the degree of inhibition of the primary mesomeres and the extent of proliferation of mesenchyme cells from the epidermis of the foot and body may cause variation. An additional contributing cause may be impairment of the larval kidneys, which appear, at least in *Physa*, to be derivatives of the two primary mesomeres (Wierzejski, 1905).

Similarly, heavy metal ions cause exogastrulation and hydropic larvae in echinoderms, partly by inhibiting the mesenchyme-forming regions. Hydropic larvae with isolated mesenchyme cells and abnormal spicules occur (Mateyko, 1961; Lallier, 1956; Rulon, 1955). Radial larvae and polar-elongated larvae, described

by Rulon (1955) and Lallier (1956), may also result from abnormal differentiation of the mesenchyme cells.

Arrested larvae

The normal *Limnaea* embryo incorporates capsule fluid by pinocytosis as early as the 40-cell stage (Raven, 1946; Elbers and Bluemink, 1960); ectodermal incorporation of capsule fluid continues after gastrulation and is progressively localized in the velar cells and large cells of the head vesicle (Raven, 1946). During the third day of development the esophageal process of the stomodeum coalesces with the mid-gut and the capsule fluid is actively ingested *via* the digestive tract. The ectoderm cells no longer stain for capsule fluid and the larval liver cells become the main sites of capsule fluid digestion (Raven, 1946, 1958; Bloch, 1938). Apparently cobalt elicits arrested larvae and shell-less snails by inhibition of liver cells. The resultant inadequate utilization of the capsule fluid eventually starves these larvae. Although the small cobalt-treated larvae had only a few, irregularly stained liver cells, they lived for several days. Evidently their larval liver was able to sustain minimal metabolism but not support growth. Variation in the functional capacity of the larval livers may have caused the observed differences in development of arrested veligers.

Mechanism of action of cobalt

Our current ignorance of the structure and function of cells and of the nature of embryonic development makes interpretation of the cobalt effect difficult. The biological effect of metal ions is undoubtedly due either to their combination with free anionic groups of functional significance or to replacement of biologically critical cations. In the case of *Limnaea*, the site of action must lie in the fourth quartette of micromeres and persist in their derivatives, the mesomeres and larval liver cells.

Non-uniform distribution of functional groups has been shown in *Limnaea* eggs and other organisms. Raven (1946) found non-uniform distribution of bound $-SH$ groups in the gastrula and trochophore stages of *Limnaea*. Fauré-Fremiet and Mugard (1948) observed in *Teredo* eggs cortical localization of an argyrophilic substance, perhaps lipoprotein in nature, which appeared to be related to detergent-sensitivity of these eggs. Differentiating echinoderm eggs showed progressive segregation of reactive groups (Immers, 1961, 1962; Bäckström, 1957, 1961). Our results support the idea that there is a cobalt-sensitive macromolecule, located in the vegetal region of the *Limnaea* egg, which is progressively segregated into the large-celled endomeres and mesomeres.

Until further studies are made, the nature of the macro-molecule can only be hypothetical. Immers' studies (1961, 1962) suggest sulfated polysaccharides as likely sites of metal-reactivity. Cobalt is known to mask $-SH$ groups of free and protein-bound cysteine (Summer and Somers, 1953; Kinoshita, 1955; Gurd and Wilcox, 1956) and to complex with amino groups of free and bound basic amino acids (Lehninger, 1950; Williams, 1953). It is equally possible that cobalt acts by displacement of critical cations; Raven (1956) and Geilenkirchen (1961) have suggested that the biological effects of high concentrations of Li^+, Na^+ and K^+ on *Limnaea* may be due to displacement of calcium.

It is easy to postulate the existence of cobalt-reactive groups in molecules critical to development; the problem of the future is to identify such receptor sites and to understand their role in normal development.

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Summary

1. The effect of cobaltous chloride ($CoCl_2$) on the developmental stages of *Limnaea stagnalis* and *L. palustris* has been studied.

2. Eggs and embryos were treated for 24 hours with various concentrations of $CoCl_2$. Effective concentrations of $CoCl_2$ ranged from 1×10^{-5} to 1×10^{-4} *M* for *L. stagnalis* and from 2×10^{-6} to 8×10^{-5} *M* for *L. palustris*.

3. Variations in resistance to $CoCl_2$ were observed between species at the same stage of development, between eggs treated at different stages of development and between eggs from the same egg masses treated at the same stage of development.

4. Treatment with $CoCl_2$ resulted in (a) separation of blastomeres, (b) vesicular and dumbbell-shaped exogastrulae, (c) arrested gastrulae, (d) veligers with a reduced larval liver, shell and foot, and with a fluid-filled body cavity containing few mesenchyme cells, (e) shell-less snails with a reduced larval liver and shell, and (f) helmet-shelled snails with abnormally wide shell apertures.

5. Manganese, cadmium and nickel produced malformations similar to those caused by cobalt. The sequence of effective concentrations was Mn < Co < Cd < Ni.

6. Sections of normal and cobalt-treated embryos showed an inhibition of the differentiation and proliferation of mesenchyme cells of the body and foot and an inhibition of proliferation and enlargement of the endoderm cells of the larval liver.

7. Exogastrulae and hydropic exogastrulae and veliger larvae appear to be caused by an impairment in the development of the mesomeres plus a concomitant uptake of water by the blastocoel or body cavity.

8. Arrested veliger larvae and the differences in their development are explained on the basis of variation in the functional capacity of their larval livers.

9. The morphological evidence indicates that the several characteristic malformations result from an inhibition of the cobalt-sensitive fourth quartette of micromeres and their derivatives, the mesomeres and larval liver cells.

10. The results suggest that metal ion-reactive groups in the vegetal region of the *Limnaea* egg may be progressively segregated into the fourth quartette of micromeres.

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